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Elucidation of the quaternary structure of the insulin receptor

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Photoreactive insulin analogues specifically label predominantly one polypeptide in the insulin receptor of rat liver plasma membranes. We have used the bifunctional reagent disuccinimidyl suberate to cross-link this polypeptide to its neighbouring, but not necessarily labelled, subunits. The results of these studies show that (1) there are at least three types of subunit in the receptor, with apparent M_r ($M_{app.}$) values of 65000, 95000 and 120000; (2) the receptor appears to consist of two $M_{app.}$ 120000, one $M_{app.}$ 95000 and one $M_{app.}$ 65000 subunits; (3) the $M_{app.}$ 65000 subunit, which has not been previously reported, may be only loosely attached to the receptor, and does not interact directly with the insulin-binding subunit ($M_{app.}$ 120000).

Insulin stimulates the cells upon which it has an effect by binding to a plasma membrane receptor (Cuatrecasas, 1969). The elucidation of the transduction of this binding event into an intracellular stimulus will require a detailed knowledge of the structure of the receptor. Methods used to study the constituent polypeptides of the receptor have included affinity labelling techniques (Yip *et al.*, 1978; Wisher *et al.*, 1980; Pilch & Czech, 1980), immunoprecipitation with antibodies to the receptor (Jacobs *et al.*, 1980; Harrison & Itin, 1980), biosynthetic labelling of protein (Van Obberghen *et al.*, 1981) or carbohydrate (Hédo *et al.*, 1980) parts of the receptor, target size analysis (Harmon *et al.*, 1981; Pollet *et al.*, 1982), and various methods of receptor purification (Jacobs *et al.*, 1977; Williams & Turtle, 1979; Harrison & Itin, 1980; Meyer *et al.*, 1981). The consensus of these studies is that there are several different polypeptides in the native receptor, one of which [apparent molecular weight ($M_{app.}$) 120000–130000] contains the insulin-binding site. There is no agreement, however, on the numbers of each subunit in the native receptor. Current hypotheses are based primarily on observations of affinity-labelled high-molecular-weight forms of the receptor that are held together by interchain disulphide bonds or strong hydrophobic interactions (Jacobs *et al.*, 1980; Pilch & Czech, 1980; Baron *et al.*, 1981). These methods, however, look only at polypeptides that happen to have a natural covalent bond, or close hydrophobic link, with the insulin binding subunit.

Abbreviation used: SDS, sodium dodecyl sulphate.

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We have found (Wisher *et al.*, 1980) that photoreactive insulin analogues label predominantly one polypeptide in the liver plasma membrane insulin receptor, which corresponds to the insulin binding subunit. The insulin receptor can be solubilized with almost full ability to bind insulin by the non-ionic detergent Triton X-100 (Cuatrecasas, 1972), indicating that the detergent-solubilized protein largely retains its native conformation. We report here that the solubilized, photoaffinity-labelled, receptor can be cross-linked with the bifunctional reagent disuccinimidyl suberate to produce a number of discrete, covalently linked, oligomers. From the hetero-oligomers produced at different concentrations of disuccinimidyl suberate the number and size of subunits in the native receptor have been deduced.

Experimental

Highly purified rat liver plasma membranes were prepared by the method of Wisher & Evans (1975). N^{B29}-(4-Azido-2-nitrophenyl)acetyl-insulin was the gift of Drs. D. Saunders and D. Brandenburg, Deutsches Wollforschungsinstitut, Aachen, Germany; it was iodinated by the method of Roth (1975) to a specific activity of 150–200 μCi/μg and purified by the gel electrophoresis method of Linde *et al.* (1981).

The insulin receptor was photoaffinity labelled and solubilized in Triton X-100 essentially as previously described (Baron *et al.*, 1981) except that (*a*) all buffers contained phenylmethylsulphonyl fluoride (15 μg/ml), Aprotinin (200 kallikrein inhibitor units/ml) and EDTA (0.2 mM) to inhibit proteolytic enzymes, and (*b*) for cross-linking studies the Tris content of buffers was replaced with an

equimolar concentration of triethanolamine, and bovine serum albumin and EDTA were omitted.

For the preparation of liver plasma membranes in the presence of proteinase inhibitors the media used contained five times these concentrations of inhibitors.

Disuccinimidyl suberate solutions were freshly prepared in dimethyl sulphoxide. Portions (5 μl) of disuccinimidyl suberate in dimethyl sulphoxide were added to 95 μl of Triton X-100-solubilized membrane to give the appropriate final concentration of disuccinimidyl suberate, and the mixture was kept at 0°C for 15 min. The reaction was stopped by the addition of 100 μl of electrophoresis sample buffer [0.25 M-Tris/HCl (pH 6.8)/8 M-urea/2 mM-EDTA] containing 20% (w/v) SDS. Samples were boiled with or without 50 mM-dithiothreitol for 5 min.

Electrophoresis in polyacrylamide gels of the SDS-solubilized proteins was performed in the buffer system of Laemmli (1970). Stacking gels were 3% and resolving gels 5% total acrylamide; the acrylamide:bisacrylamide ratio was 82.3:1. Gels were fixed in 20% (w/v) trichloroacetic acid and stained and destained as described (Wisher *et al.*, 1980). Gels were dried and autoradiographs made with Cronex 'Hi-plus' intensifying screens and Cronex-4 X-ray film that had been pre-exposed to increase sensitivity (Laskey & Mills, 1977). Glutaraldehyde-cross-linked bovine serum albumin polymers were prepared essentially by the method of Payne (1973). The polymer mixture was stable at 4°C for up to 3 days, but was normally prepared fresh for each electrophoresis run. Albumin polymers up to the hexamer (M_r 408000) were discernible. Other molecular weight markers were myosin (M_r 200000), β-galactosidase (M_r 116500), phosphorylase b (M_r 92000) and bovine serum albumin monomer (M_r 68000) from Bio-Rad. Aprotinin was from Bayer (U.K.); disuccinimidyl suberate was from Pierce Biochemicals, and phenylmethylsulphonyl fluoride, bovine serum albumin and Tris were from Sigma. All other chemicals were from BDH and were of Analar grade, except for glutaraldehyde, which was general reagent grade.

Results and discussion

Photoaffinity-labelled insulin receptor from liver plasma membranes revealed a single major labelled polypeptide [$M_{app.}$ 120000 ± 4000 ($n = 5$); mean ± S.D.] when analysed by SDS/polyacrylamide-gel electrophoresis after reduction of disulphide bonds to permit full protein denaturation (Fig. 1). In addition a very lightly labelled band ($M_{app.}$ 95000) was also sometimes seen. Labelling of both these proteins, and all other labelled proteins seen, was completely abolished if photoaffinity labelling was carried out in the presence of 5×10^{-6} M-unlabelled insulin (Fig. 1).

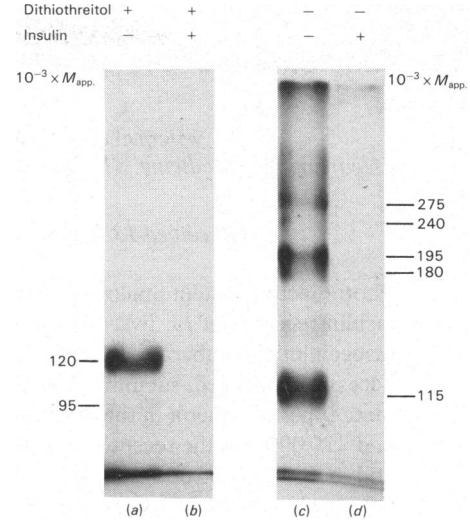

Fig. 1. *Analysis by SDS/polyacrylamide-gel electrophoresis of photoaffinity-labelled insulin receptor*
Liver plasma membranes were labelled with ^{125}I-labelled N^{B29}-(4-azide-2-nitrophenyl)acetyl-insulin in the presence (*b*, *d*) or absence (*a*, *c*) of 5×10^{-6} M-unlabelled insulin. Membranes were homogenized in sample buffer containing 10% (w/v) SDS, boiled for 5 min in the presence (*a*, *b*) or absence (*c*, *d*) of 50 mM-dithiothreitol and analysed on polyacrylamide gels as described in the Experimental section. Apparent molecular weights ($M_{app.}$) were determined by comparison with molecular weight standards that had been similarly treated (i.e. reduced or unreduced).

If disulphide bonds are not reduced, full protein denaturation cannot take place. Molecular weight estimates of non-reduced proteins from SDS/polyacrylamide-gel electrophoresis are therefore inaccurate. However, as can be seen from Fig. 1, track (*c*), higher molecular weight forms of the receptor are seen under these conditions, suggesting that the receptor subunits are held together by interchain disulphide bonds, as has been previously reported (Jacobs *et al.*, 1979; Massagué *et al.*, 1980).

Effects of cross-linking agent

Low concentrations (<1.0 mM) of disuccinimidyl suberate produced, in addition to the $M_{app.}$ 120000 band, three oligomers [$M_{app.}$ 219000 ± 4000 ($n = 5$), 248000 ± 10000 ($n = 5$) and 328000 ± 9000 ($n = 5$)] (Fig. 2*a*). At higher concentrations the $M_{app.}$ 330000 band became much more intensely labelled, and two further oligomers were produced [$M_{app.}$ 165000 ± 5000 ($n = 3$) and 393000 ± 12000 ($n = 4$)] (Fig. 2*a*). No higher molecular weight oligomers that would enter the gel were seen at concentrations of disuccinimidyl suberate up to 10 mM (results not shown). As the

concentration of disuccinimidyl suberate was increased, the amount of labelled protein remaining in the stacking gel also increased. This was probably protein that was too heavily modified by disuccinimidyl suberate to unfold in SDS, and which therefore did not enter the gel. It is also noticeable that the increase in labelled high molecular weight oligomers is accompanied by a decrease in the amount of $M_{app.}$ 120000 subunit, confirming that this polypeptide is being cross-linked into the larger complexes.

In the absence of reducing agents (Fig. 2*b*), oligomers apparently corresponding to the $M_{app.}$ 165000, 220000, 250000 and 330000 bands are seen in the uncross-linked receptor, although their relative mobilities are different, because of different disulphide content. After cross-linking more labelled polypeptide is seen in the $M_{app.}$ 395000 form, which appears to represent the full receptor. This molecular weight is considerably higher than the value of approx. 280000 obtained by hydrodynamic analysis (Baron *et al.*, 1981). Since it has been shown (Wisher *et al.*, 1980) that the anomalous migration of the insulin-binding subunit on SDS/polyacrylamide-gel electrophoresis gels leads to an overestimate (120000–130000) of its true molecular weight (90000), it is not unreasonable to assume that the value of $M_{app.}$ derived by SDS/polyacrylamide-gel electrophoresis for the whole receptor may also be an overestimate.

Effects of inhibitors of proteolytic enzymes

There is now some evidence (Massagué *et al.*, 1981; Harrison *et al.*, 1982) that the insulin receptor may be unusually susceptible to proteolytic enzymes. Membranes were therefore prepared in media containing high concentrations of proteolytic enzyme inhibitors, and the receptor labelled and cross-linked as above. Comparison of Figs. 2 and 3 shows that inhibitor-protected membrane preparations (Fig. 3) do not show the $M_{app.}$ 165000 oligomer, nor any of the lightly-labelled low-molecular-weight ($M_{app.}$ <90000) peptides. In addition, a larger proportion of the labelled receptor is cross-linked into the $M_{app.}$ 395000 form in both reduced (Fig. 3*a*) and non-reduced (Fig. 3*b*) samples. This confirms that the $M_{app.}$ 395000 form is the full receptor, and suggests that the $M_{app.}$ 165000 form may be an oligomer containing one or more proteolytic fragments of receptor peptides.

In normal membrane preparations all manipulations are performed at 0–4°C, conditions which are

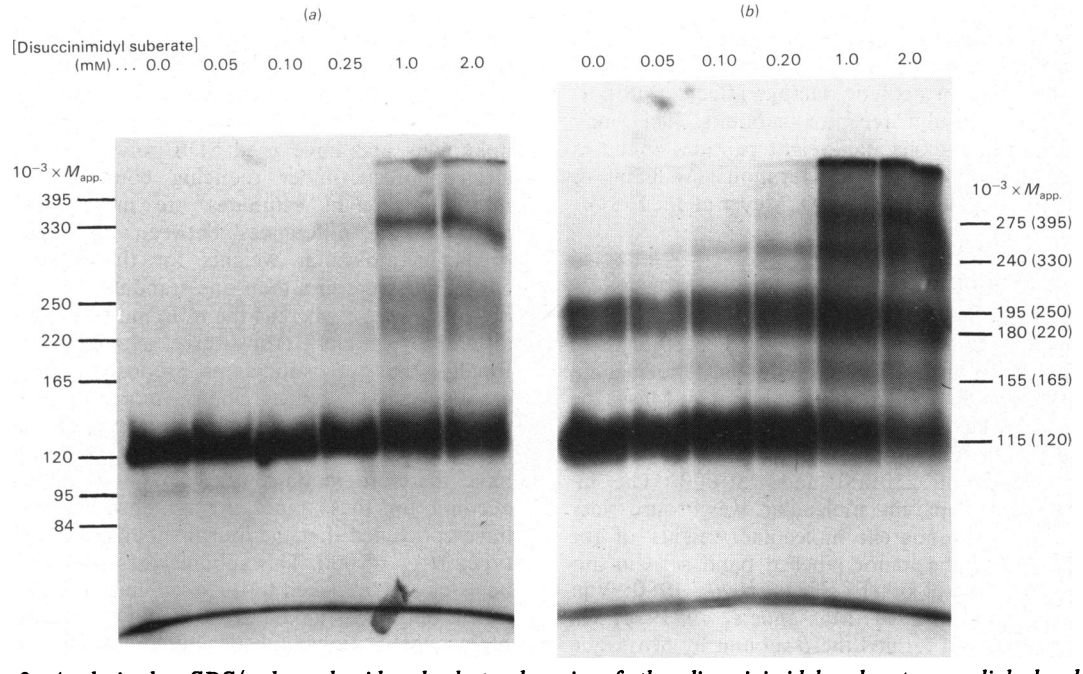

Fig. 2. *Analysis by SDS/polyacrylamide-gel electrophoresis of the disuccinimidyl suberate-cross-linked, photoaffinity-labelled, insulin receptor*

Photoaffinity-labelled liver plasma membranes were solubilized in 2% Triton X-100 and treated with the indicated concentration of disuccinimidyl suberate as described in the Experimental section. The cross-linked samples were divided and boiled for 5 min in the presence (*a*) or absence (*b*) of 50 mM-dithiothreitol. The values of $M_{app.}$ in (*b*) are those derived from unreduced markers, with the corresponding $M_{app.}$ of the oligomer when reduced, in parentheses.

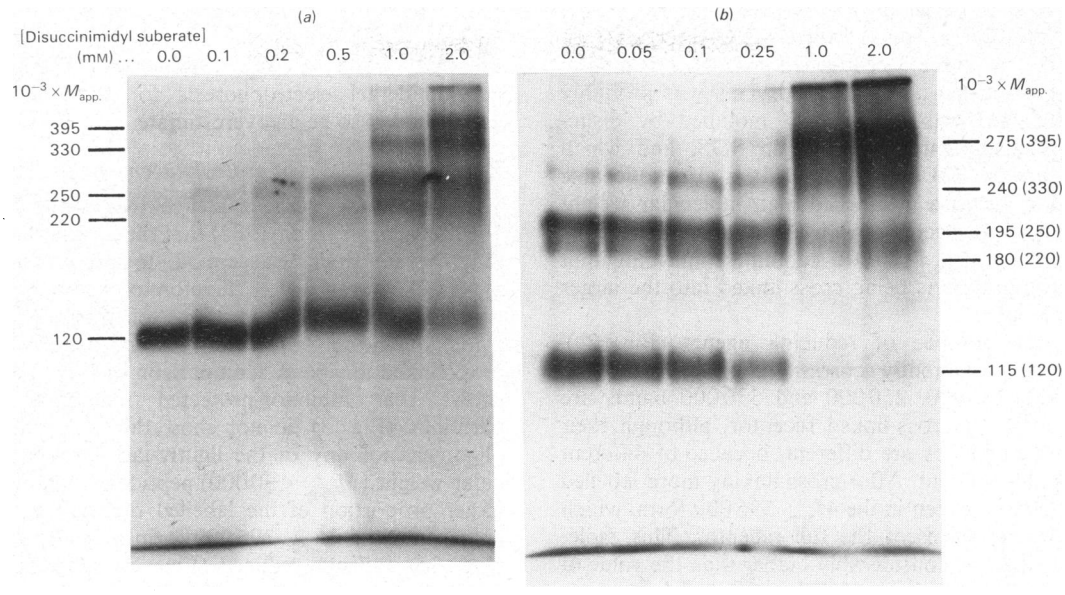

Fig. 3. *Analysis by SDS/polyacrylamide-gel electrophoresis of the disuccinimidyl suberate-cross-linked, photoaffinity-labelled, insulin receptor*

The experiment was carried out as described in the legend to Fig. 2, except that membranes were prepared in the presence of proteinase inhibitors; (*a*) and (*b*) are from separate experiments. Values of $M_{app.}$ in (*b*) are again those derived from unreduced standards, with the corresponding value for the reduced oligomer in parentheses.

normally thought to preserve the integrity of plasma membrane proteins. Under these conditions, however, some proteolytic damage affects a proportion of the insulin receptor subunits. This may explain the low-molecular-weight peptides found in several purified receptor preparations (Williams & Turtle, 1979; Lang *et al.*, 1980; Meyer *et al.*, 1981).

Receptor subunit composition

From the molecular weights of the oligomers obtained by cross-linking the labelled receptor there appear to be at least four subunits. The insulin-binding subunit [termed the α subunit by Massagué *et al.* (1981)], appears to have two neighbours, giving rise to the two-polypeptide oligomers seen at $M_{app.}$ values of 220000 and 250000. The increments in apparent molecular weight are very similar to the apparent molecular weights of the α-subunit and the minor labelled band seen in this study and several others (Hédo *et al.*, 1980; Van Obberghen *et al.*, 1981; Massague *et al.*, 1981, Yip *et al.*, 1980) and termed the β-subunit by Massagué *et al.* (1981). Cross-linking of all three polypeptides together gives rise to the $M_{app.}$ 330000 band, while the last cross-linking step produces the $M_{app.}$ 395000 band, revealing a fourth subunit. According to a previous model of the receptor (Massagué *et al.*, 1981) this should be another β-subunit, giving rise to an $M_{app.}$ 430000 oligomer. However, in this study, as opposed to those upon which this earlier model was based, we have used molecular weight markers spanning the range of molecular weights of the unknowns and have used SDS/polyacrylamide-gel electrophoresis under reducing conditions, when molecular weight estimates are more valid. In addition, the differences between observed and predicted molecular weights for the other three complexes were less than one standard error of the observed $M_{app.}$, whereas the $\alpha_2\beta_2$ model produces a difference greater than three standard errors. Finally, the $\alpha_2\beta_2$ model, as proposed (Massagué *et al.*, 1981) predicts many more cross-linked complexes than were observed, especially in the non-proteinase-protected preparation (Fig. 2). We have therefore modified their initial hypothesis to account for these more precise observations, and have concluded that the fourth subunit is a different type, $M_{app.}$ 65000. This subunit does not appear to be as closely attached to the α-subunit as the others, as it is only cross-linked to the rest of the receptor by high concentrations of disuccinimidyl suberate.

The presence of multi-subunit forms of the receptor, even after boiling in SDS, if disulphide bonds are not reduced, has led to the speculation (Jacobs *et al.*, 1979; Massagué *et al.*, 1981) that the receptor subunits are linked by disulphide bonds. This hypothesis does not take into account the

observations made by several groups (Ginsberg *et al.*, 1976; Maturo & Hollenberg, 1978; Krupp & Livingston, 1979; Baron *et al.*, 1981) that the insulin receptor subunits can be dissociated and reassociated without disulphide reduction. In our studies here, most of the receptor appears as single polypeptide or two-polypeptide complexes, even without disulphide reduction. It is possible that the subunits, if prevented from fully denaturing by the presence of intrachain disulphide bonds, may be held together by SDS-resistant hydrophobic interactions, as is not uncommon for membrane proteins (e.g. Katzman, 1972; Silverberg & Marchesi, 1978; Hendersen *et al.*, 1979; Argyrondi-Akoyunoglou & Thomou, 1981);

Several studies have identified an M_r 40000–50000 subunit as a component of the insulin receptor (Jacobs *et al.*, 1979, 1980; Harrison & Itin, 1980; Pollet *et al.*, 1982; Yip *et al.*, 1982). Other studies have suggested that this 'subunit' is a proteolytic fragment of the β-subunit (Massagué *et al.*, 1981). We have found that membranes that have not been fully protected from possible proteolysis during preparation contain an $M_{app.}$ 45000 peptide that is cross-linked to the α-subunit to give the $M_{app.}$ 165000 oligomer. Such mild proteolysis does not seem to affect the functional integrity of the receptor (Pilch *et al.*, 1981). The decrease in the amount of $M_{app.}$ 395000 oligomer due to proteolysis may not be due to loss of the $M_{app.}$ 65000 subunit (δ-subunit), which is still present in the unreduced receptor (Fig. 2*b*), but to failure to cross-link the δ-subunit to the α-subunit after cleavage of the β-subunit. This, and the lack of any α–δ oligomer, suggests that the δ-subunit is cross-linked to the β-subunit rather than to the α-subunit. Previous attempts at receptor purification have not co-purified an $M_{app.}$ 65000 subunit. The methods used, however, have either involved strongly dissociating media (Jacobs *et al.*, 1977; Harrison & Itin, 1980) or have utilized lectin affinity chromatography, a method specific for glycoproteins (that is, proteins exposed at the cell surface). If the δ-subunit is not exposed at the outside of the cell, and is not covalently linked to the rest of the receptor, it may be lost when the other subunits are retained on the affinity column. It is interesting to note that an M_r 68000 peptide has recently been reported as being co-precipitated with other receptor subunits by specific anti-receptor antibodies (Kasuga *et al.*, 1982), but is not seen if the receptor is pre-purified on a wheatgerm agglutinin–Sepharose column.

These ideas are illustrated in Fig. 4 which, we must emphasize, is not a proposed model of the receptor, but a diagramatic representation of our current working hypothesis. Further elucidation of the orientation of the δ-subunit to the rest of the receptor, and the function of the different polypeptides, will require study of the receptor *in situ*, in its membrane.

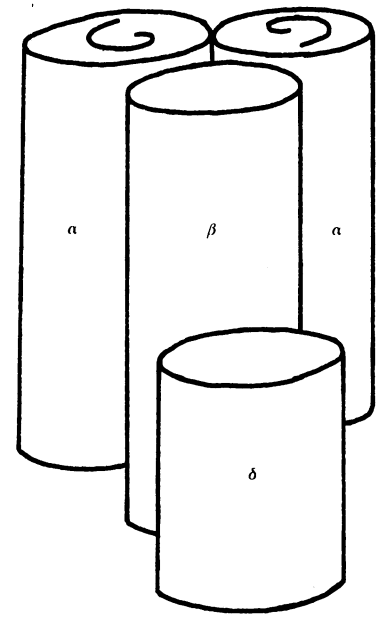

Fig. 4. *Current hypotheses of insulin receptor structure*
The insulin receptor appears to consist of two insulin binding subunits ($M_{app.}$ 120000; α) and one each of the non-binding subunits β ($M_{app.}$ 95000) and δ ($M_{app.}$ 65000). This last subunit probably does not face the outside of the cell, as it does not appear to be a glycoprotein.

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References

Argyroudi-Akoyunoglou, J. & Thomou, H. (1981) *FEBS Lett.* **135**, 177–181

Baron, M. D., Wisher, M. H., Thamm, P. M., Saunders, D. J., Brandenberg, D. & Sönksen, P. H. (1981) *Biochemistry* **20**, 4156–4161

Cuatrecasas, P. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **63**, 450–457

Cuatrecasas, P. (1972) *J. Biol. Chem.* **247**, 1980–1991

Ginsberg, B. H., Kahn, C. R., Roth, J. & De Meyts, P. (1976) *Biochem. Biophys. Res. Commun.* **73**, 1068–1074

Harmon, J. T., Kempner, E. S. & Kahn, C. R. (1981) *J. Biol. Chem.* **256**, 7719–7722

Harrison, L. C. & Itin, A. (1980) *J. Biol. Chem.* **255**, 12066–12072

Harrison, L. C., Itin, A., Kasuga, M. & Van Obberghen, E. (1982) *Diabetologia* **22**, 233–238
Hédo, J. A., Kasuga, M., Van Obberghen, E., Roth, J. & Kahn, C. R. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4791–4795
Hendersen, D., Eibl, H. & Weber, K. (1979) *J. Mol. Biol.* **132**, 193–218
Jacobs, S., Shecter, Y., Bissell, K. & Cuatrecasas, P. (1979) *Biochem. Biophys. Res. Commun.* **77**, 981–988
Jacobs, S., Hazum, E., Shecter, Y. & Cuatrecasas, P. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4918–4921
Jacobs, S., Hazum, E. & Cuatrecasas, P. (1980) *J. Biol. Chem.* **255**, 6937–6940
Kasuga, M., Karlsson, F. A. & Kahn, C. R. (1982) *Science* **215**, 185–186
Katzman, R. L. (1972) *Biochim. Biophys. Acta* **266**, 269–272
Krupp, M. N. & Livingstone, J. N. (1979) *Nature (London)* **278**, 61–62
Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
Lang, U., Kahn, C. R. & Harrison, L. C. (1980) *Biochemistry* **19**, 64–70
Laskey, R. A. & Mills, A. D. (1977) *FEBS Lett.* **82**, 314–316
Linde, S., Hansen, B., Sonne, O., Holst, J. J. & Gliemann, J. (1981) *Diabetes* **30**, 1–8
Massagué, J., Pilch, P. F. & Czech, M. P. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7137–7141
Massagué, J., Pilch, P. F. & Czech, M. P. (1981) *J. Biol. Chem.* **256**, 3182–3190
Maturo, J. M. & Hollenberg, M. D. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3070–3074
Meyer, H. E., Bubenzer, H.-J., Herbertz, L., Kuehn, L. & Reinaur, H. (1981) *Hoppe-Seyler's Z. Physiol. Chem.* **362**, 1621–1629
Payne, J. W. (1973) *Biochem. J.* **135**, 867–873
Pilch, P. F. & Czech, M. P. (1980) *J. Biol. Chem.* **255**, 1722–1731
Pilch, P. F., Axelrod, J. D., Collelo, J. & Czech, M. P. (1981) *J. Biol. Chem.* **256**, 1570–1575
Pollet, R. J., Kempner, E. S., Standaert, M. L. & Haase, B. A. (1982) *J. Biol. Chem.* **257**, 894–898
Roth, J. (1975) *Methods Enzymol.* **37**, 223–233
Silverberg, M. & Marchesi, V. T. (1978) *J. Biol. Chem.* **253**, 95–98
Van Obberghen, E., Kasuga, M., Le Cam, A., Hédo, J. A., Itin, A. & Harrison, L. C. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1052–1056
Williams, P. F. & Turtle, J. R. (1979) *Biochem. Biophys. Acta* **579**, 367–374
Wisher, M. H. & Evans, W. H. (1975) *Biochem. J.* **146**, 375–388
Wisher, M. H., Baron, M. D., Jones, R. H., Sönksen, P. H., Saunders, D. J., Thamm, P. & Brandenburg, D. (1980) *Biochem. Biophys. Res. Commun.* **92**, 492–498
Yip, C. C., Yeung, C. W. T. & Moule, M. L. (1980) *Biochemistry* **19**, 70–76
Yip, C. C., Yeung, C. W. T. & Moule, M. L. (1978) *J. Biol. Chem.* **253**, 1743–1745
Yip, C. C., Moule, M. L. & Yeung, C. W. T. (1982) *Biochemistry* **21**, 2940–2945